

INTERACTIONS BETWEEN SURFACTANT ALKYL SULFO-N-SUCCINIMIDYL ESTERS AND COLLAGEN FIBRILS

by

C.E. KUNG*, B.E. MALEEFF**, R.L. DUDLEY AND P.L. KRONICK***

U.S. Department of Agriculture, Agricultural Research Service

Eastern Regional Research Center

600 EAST MERMAID LANE

PHILADELPHIA, PENNSYLVANIA 19118

Abstract

A surface-active agent, sulfo-N-succinimidyl undecenoate (SSU) was prepared as a probe for collagen-surfactant interactions. When the probe was mixed with monomeric and dimeric collagen in solution, the α_1 and β_{11} chains reacted to give an electrophoretically homogenous product with increased mobility; the mobilities of the α_2 and β_{12} chains were unaltered. The surfactant appeared to coat fibrils of collagen when dried and viewed in the electron microscope. Experimental evidence for the chemical reactivity of SSU with hippuryllsine under mild conditions was obtained by NMR. Besides serving as a probe for investigations, SSU might also be a model of a permanent leather lubricant requiring minimal amounts of material.

Introduction

Fatliquoring is the last wet chemical step in the leathermaking process^{1,2}. Presently, traditional fatliquoring reagents are being replaced with more biodegradable chemicals. This means searching for new hydrophobic and oleophobic rendering agents, and alternatives to the alkyl phenols and organic halogen compounds currently used³. As the term implies, fatliquoring is the application to leather of mixtures of oils and emulsifiers. Fatliquors may be either anionic, cationic or neutral and can have varying degrees of sulfonation and hydrophobic character^{1,2}. Sulfonation enhances metallic ion interactions and miscibility with water. Fatliquor impregnation of leather lubricates the fibers within leather, keeping them from adhering to each other^{1,2}. As a consequence, the leather acquires softness and a degree of waterproofing. Alternatives to fatliquors are available, but they seem to require the presence of chromium to bind them to leather⁴.

Surface active agents (surfactants) are amphiphilic molecules that are capable of undergoing self-assembly into aggregated systems such as monolayers (e.g., micelles) and bilayers (e.g., vesicles)⁵⁻⁷. These unique molecules generally consist of a polar head group (neutral or charged) and one or two hydrophobic tail(s). As a consequence of the hydrophobic effect, the surfactants within the aggregated assemblies are oriented with their polar headgroups exposed to the aqueous phase while their hydrophobic tails face away from the aqueous phase, thereby forming a hydrophobic core⁷. Extensive analyses of the dynamic and physical properties of amphiphiles and their aggregated structures have been conducted⁵⁻¹⁷.

Reich¹⁸ has shown that the amount of surfactant that must be applied to leather to achieve the desired product properties exceeds by tenfold the amount calculated to coat the collagen fibrils with a monomolecular layer, although these properties were retained when most but not all the

* Present address, Washington University, St. Louis, MO.

** Present address SmithKline Beecham Pharmaceuticals, Philadelphia, PA.

*** Correspondence should be addressed to P.L. Kronick.

applied material was extracted. Evidently the spatial distribution of the surfactant when it is applied has yet to be optimized. It was the aim of this work to use reactive probes to determine where a detergent binds to collagen fibrils. Amphiphilic molecules were prepared that would react covalently with ϵ -amino groups of the lysine residues of collagen and that would also act like a fatliquor in coating the surfaces of the fibrils. These reagents also contained double bonds so that, by polymerizing, an amplification might be achieved and their presence and locations observed experimentally. The compounds investigated were alkyl sulfo-N-succinimidyl esters (I) (see Fig. 1).

In the general structure, the R₁ moiety, which is derived from an unsaturated carboxylic acid, provides the hydrophobic character and the functionality for polymerization. The alkenoic acid used here is 10-undecenoic acid (11:1). The heterocyclic ring system is the N-hydroxysulfosuccinimide group. This group carries the negative charge and thus represents the polar head group of the surfactant. In addition (as discussed below), it is the leaving group when the surfactant reacts with collagen. The esters (I) are amphiphilic and are similar in structure to the sodium alkanesulfonates. This paper will discuss the synthesis of these compounds and will report on their reactions with hippuryllysine and fibrillar collagen.

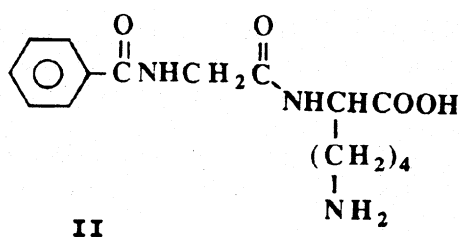
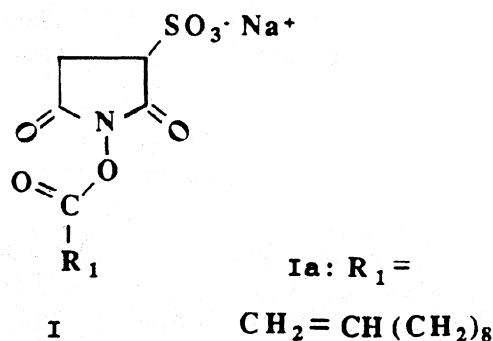


FIG. 1. — The chemical structures for alkyl sulfo-N-succinimidyl ester (I), SSU (Ia) and hippuryl lysine (II).

Experimental

METHODS

All reagents used are of ACS Certified grade or better. N-Hydroxymaleimide, sodium metabisulfite, N,N-dimethylformamide and 1, 3-dicyclohexylcarbodiimide* were purchased from Aldrich Chemical Co., Inc.** N-(2-Hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid) (Hepes) was obtained from the Sigma Chemical Co. Acid-soluble collagen obtained from Pentapharm Ltd. (Centerchem, Inc.) was used without further purification.

N,N-dimethylformamide was vacuum-distilled (76° C, 39 Torr) and stored over 4 Å Molecular Sieves as per Vogel¹⁹. Purified water produced from a Modular Lab Polisher I (HPLC) system (Combined Water Systems Corporation) was routinely used. Hippuryllysine (II) and 10-undecenoic acid were obtained from Cyclo Chemical Corp. and Eastman Organic Chemicals Inc., respectively. Deuterated dimethylsulfoxide (DMSO-d₆, 99.5% isotopic purity) was obtained from Merck Sharpe & Dohme Canada Ltd. Electrophoretic grades of acrylamide, N,N'-methylene-bis-acrylamide, ammonium persulfate, sodium dodecyl sulfate (SDS) and N,N,N',N'-tetramethylethylenediamine were obtained Bio-Rad Laboratories. Tris(hydroxymethyl)aminoethane, Coomassie Brilliant Blue R-250 and glycine were purchased from the Sigma Chemical Co.

SYNTHESIS OF POLYMERIZABLE SURFACE ACTIVE AGENT

The synthetic route was adapted from that of Staros²⁰ for the preparation of alkyl sulfo-N-succinimidyl esters from carboxylic acids.

PREPARATION OF N-HYDROXYSULFOSUCCINIMIDE

N-Hydroxymaleimide (1.45 g, 12.8 mmol) was dissolved in absolute ethanol (20 mL) and mixed under dry nitrogen at room temperature with a 10 mL aqueous solution of sodium metabisulfite (1.22 g, 6.44 mmol). After stirring for 2 hours, the reaction mixture was exposed to air, and the solvent was evaporated under vacuum. The residual yellow oil was dissolved in 50 mL of water and lyophilized. The solid was extracted with ethyl ether and recrystallized from 80-90% aqueous ethanol. The crude off-white solid (1.1 g, 50% yield) obtained here was used below.

COUPLING TO AN UNSATURATED CARBOXYLIC ACID

Under dry nitrogen, 10-undecenoic acid (2.2 mmol, 0.40 g), N-hydroxysulfosuccinimide (2 mmol, 0.43 g) and 1, 3-dicyclohexylcarbodiimide (2.2 mmol, 0.45 g) were mixed with dry N,N-dimethylformamide (5 mL). The mixture was stirred at room temperature for 24 hours. The reaction was then stirred over ice water for 3 hours to precipitate the urea byproduct. The white urea solid was separated by filtration and washed with a small amount of dry N,N-dimethylformamide. This wash and the supernatant were combined. The product was precipitated from the combined supernatant by the addition of ethyl acetate (20X volume of N,N-dimethylformamide). The off-white solid was filtered and stored under vacuum. The product may turn yellow upon prolonged exposure to air. The yield of sulfo-N-succinimidyl undecenoate (SSU; Ia) was 73% (0.56 g, mp = 226-228° C). Anal. calcd. for C₁₅H₂₂O₇NSNa: C 46.99, H 5.74, N 3.66, S 8.36, Na 6.01; found; C 44.87, H 5.59, N 3.59, S 8.56, Na 6.34.

* 1,3 dicyclohexylcarbodiimide is a highly toxic and corrosive reagent.

** Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of similar nature not mentioned.

REACTION WITH THE MODIFIED AMINO ACID, HIPPURELLYSINE

At room temperature, excess hippurylllysine (II, 0.32 mmol, 0.10 g) was dissolved in 50 mL of a 20 mM Hepes, pH 7.4 solution. SSU (Ia, 0.20 mmol, 0.070 g) was vigorously mixed with the amino acid and shaken at room temperature overnight. The solution was then acidified with concentrated HCl. If no precipitation occurred, the solution was lyophilized. Otherwise the precipitate was filtered and collected. The solid was subsequently extracted with chloroform or a 70-80% chloroform/methanol mixture. After all the washes were collected, the solvent was evaporated under vacuum, and the solid residue was analyzed by ^{13}C NMR spectroscopy.

PREPARATION OF COLLAGEN FIBRILS

Collagen fibrils were prepared according to the method of George and Veis²¹. A 1 mg/mL collagen solution was prepared from a 10 mg/mL stock solution. The acid-soluble collagen was then dialyzed at 4° C against a 5 mM acetic acid solution for 3 days. Collagen samples (0.2 mg/mL) were prepared by diluting the 1 mg/mL solution with 2X phosphate buffer solution containing 18.4 mM Na_2HPO_4 , 11.6 mM KH_2PO_4 and 0.30 M NaCl (pH 7.04). Fibril formation was induced by incubating the samples without stirring at 30° C overnight in a thermostatted water bath.

REACTION OF COLLAGEN FIBRILS AND MONOMERIC COLLAGEN WITH SURFACE ACTIVE AGENTS

To a solution of the prepared fibrils (4 mL, 0.2 mg/mL collagen), SSU (Ia) was introduced until a final concentration of 0.6 mM was reached. The solution was then mixed and left standing overnight at room temperature. Aliquots of the samples were then removed for transmission electron microscopy.

Monomeric collagen (1 mg/mL) in phosphate buffer (62 mM Na_2HPO_4 and 14 mM KH_2PO_4 , pH 7.04) was prepared from Pentapharm collagen as per George and Veis²¹. Two-milliliter volumes of monomeric collagen were mixed thoroughly with 1.0 mg SSU (Ia) and left standing overnight at room temperature. Duplicate samples were lyophilized and extracted with 3X 3 mL of either ether or chloroform/methanol (2:1). After low speed centrifugation, the solvent was carefully collected with a Pasteur pipette and the samples dried with a stream of nitrogen. The controls and solvent-extracted samples were then analyzed using SDS polyacrylamide gel electrophoresis.

^{13}C NMR SPECTROSCOPY

^{13}C NMR spectra were routinely collected on either a JEOL GX-400 spectrometer operating at a frequency of 100.4 MHz or a Bruker MSL-300 spectrometer operating at 75.5 MHz, both using bi-level decoupling. For all spectra, a sweep width of 25 kHz and a recycle time of 3 sec. were used. Data (32K points) were collected and no zero-filling was performed before the Fourier transformation. All samples were dissolved in deuterated dimethylsulfoxide (DMSO-d_6), and all chemical shifts were reported in ppm using tetramethylsilane (TMS) as an external reference.

TRANSMISSION ELECTRON MICROSCOPY OF COLLAGEN SAMPLES

Microaliquots of each sample were placed on carbon-coated grids and prepared for microscopy by one of the following methods. For positive staining, specimens were treated with aqueous 1% phosphotungstic acid, pH 4.5, followed by a brief rinse with 0.1M acetic acid. Negatively stained samples were treated with aqueous 1% phosphotungstic acid, pH 7.4. All samples were examined with a Zeiss EM-10B transmission electron microscope operating at 60 kV.

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS polyacrylamide gel electrophoresis was performed following Laemmli²² on a Protean II slab cell system (Bio-Rad Laboratories). The gels used were homogeneous 4% acrylamide gels. The gels were run at 25 mA (constant current) for 5-6 hrs. and stained with a 0.1% (w/v) solution of Coomassie Brilliant Blue in methanol/acetic acid (40:10). Gel destaining was performed with a solution of methanol/acetic acid (40:10). After destaining, the gels were dried for 45 min. at 80° C using a Bio-Rad slab gel drier (Bio-Rad Laboratories).

Results

SYNTHESIS OF SURFACTANT REAGENTS

The two-stage synthesis of the alkyl sulfo-N-succinimidyl esters resulted from the sulfitolysis of N-hydroxymaleimide²³ followed by coupling to a carboxylic acid using 1,3-dicyclohexylcarbodiimide²⁴. The overall synthetic route is straightforward and high yields are obtainable. The products were relatively easy to isolate, and characterization was achieved through the peak analysis²⁵⁻²⁷ of the ¹³C NMR spectrum shown in Figure 2.

The ¹³C NMR spectra of the alkyl sulfo-N-succinimidyl esters (I) are modified composites of the spectra for N-hydroxysulfosuccinimide and the respective acid. The ¹³C NMR spectrum of N-hydroxysulfosuccinimide has four distinct resonance peaks: 30.6 ppm ($\underline{\text{C}}\text{H}_2$), 56.2 ppm ($\underline{\text{C}}\text{-C}\text{SO}_3^-$), 168.1 ppm ($\underline{\text{C}} = \text{O}$, β to $\underline{\text{C}}\text{-SO}_3^-$) and 171.4 ppm ($\underline{\text{C}} = \text{O}$). A typical ¹³C NMR spectrum for an acid has resonance peaks at 180 ppm ($\underline{\text{C}} = \text{O}$) and in the ranges: 90-140 ppm ($\underline{\text{C}} = \underline{\text{C}}$) and 14-30 ppm ($\underline{\text{C}}\text{H}_2$, $\underline{\text{C}}\text{H}_3$)^{26,27}.

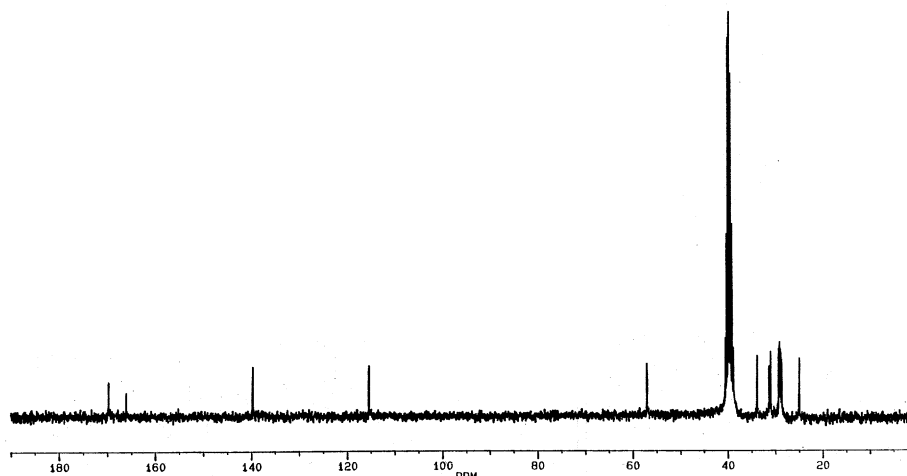


FIG. 2. — The ¹³C NMR spectrum of sulfo-N-succinimidyl undecenoate (Ia).

The ^{13}C NMR spectrum of SSU (Fig. 2) can be described as follows. The coupling reaction resulted in the loss of the free acid, and thus there was no longer a peak at 174.4 ppm (i.e., the carbonyl peak for a free acid in DMSO-d_6). Of the two peaks in the carbonyl region of the spectrum of SSU (Fig. 2), the larger and broader peak at 169.6 ppm is assigned to the ester carbonyl and the ring carbonyl farthest from the sulfonate group. Alkyl group substitution α to the carbonyl delocalizes the partial positive charge on the carbonyl carbon and results in an upfield shift of the carbonyl resonance peak²⁵⁻²⁷. Thus the nitrogen-modified ester would be expected to have a peak near 170 ppm, as observed here (169.6 ppm). The ring carbonyls of the sulfosuccinimide moiety in SSU are shifted slightly upfield from the above values; i.e., the carbonyl farthest from the sulfonate has a resonance value of 169.6 ppm compared to 171.4 ppm, and the carbonyl β to the sulfonate group has a value of 165.9 ppm, as compared to 168.1 ppm.

The terminal vinyl moiety has two asymmetric carbons and thus there are two distinct resonance peaks: one located at 115.3 ppm denoting the terminal methylene carbon and the other at 139.5 ppm for the methine carbon. At 56.9 ppm is the resonance peak for the carbon attached to the sulfonate group. The methylene carbon in the heterocyclic ring has a resonance value of 30.8 ppm. The methylene groups in the acyl chain have peaks falling in the range 24.8-33.7 ppm.

COVALENT COUPLING WITH THE MODIFIED AMINO ACID: HIPPURYLlysINE

Hippuryllysine (Fig. 1, II) was derived from lysine by modifying the α -amino group with hippuric acid. This directs subsequent reactions to the ϵ -amino group. The coupling reaction of interest here follows the reaction scheme shown in Figure 3. Nucleophilic attack by the ϵ -amino nitrogen on the acyl carbonyl carbon results in acylation of the ϵ -nitrogen of the R-group. The sodium salt of N-hydroxysulfosuccinimide acts as the leaving group. This reaction was carried out at pH 7.4 and room temperature. The products were isolated and characterized by ^{13}C NMR spectroscopy.

To determine if the coupling action occurred with hippuryllysine, the carbonyl peaks and the peaks associated with the methylene groups in the R-group of hippuryllysine were examined. Tables I and II compare the ^{13}C peak resonance values before and after the reaction for the above moieties. It is noted that in the case of the carbonyl peaks for the SSU amino acid derivative, the carbonyl associated with the new amide bond has shifted to a value of 174 ppm (see Table I).

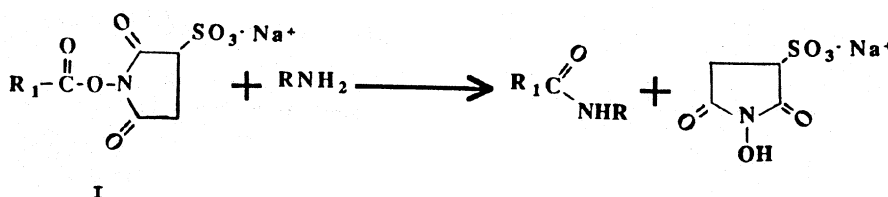


FIG. 3. — The reaction scheme for the covalent coupling of alkyl sulfo-N-succinimidyl esters (I) to amino acids containing a primary amine.

This value compares well with those reported for other alkyl substituted amide compounds²⁷.

Table II compares the peak resonance values for the R-group methylenes. In comparison to the control, all the R-group carbon peaks in the modified amino acids are shifted downfield to higher values. In particular, the shift in the α -carbon is associated with the conversion of the primary amine to a secondary amine²⁷. However, the trend in the peak values remains unchanged from the control; i.e., the minimum value at carbon position 3 is bracketed by larger values, with the largest value located at the α -carbon position. The above results indicate the new amide bond formed between the amino acid and the activated ester.

TABLE I
¹³C PEAK RESONANCE VALUES FOR THE CARBONYLS IN MODIFIED
HIPPURYLLYSINE (II)

<u>Peak Resonance Values (ppm)^a</u>		
<u>Carbonyl^b</u>	<u>Control^c</u>	<u>SSU (Ia) Derivative</u>
Ph <u>C</u> ONH	168.6	169.9
NHCH <u>C</u> OOH	174.8	174.0
CH ₂ <u>C</u> ONHCH	167.8	168.0
CH ₂ <u>C</u> ONHCH ₂	—	174.0

^aThe spectra were acquired as per Materials and Methods.

^bThe underlined carbons denote the ones with the given peak resonances.

^cThe control is the unmodified form of hippuryllysine (II).

TABLE II
¹³C PEAK RESONANCE VALUES FOR THE METHYLENE GROUPS ADJACENT
TO THE ϵ -AMINO GROUP OF MODIFIED HIPPURYLLYSINE (II).

<u>Peak Resonance Values (ppm)^a</u>		
<u>Methylene^b</u>	<u>Control^c</u>	<u>SSU (Ia) Derivative</u>
<u>CH</u> ₂ (4)	32.1	36.0
<u>CH</u> ₂ (3)	22.1	25.7
<u>CH</u> ₂ (2)	27.2	31.2
<u>CH</u> ₂ (1)	43.5	49.7

^aThe spectra were acquired as per Materials and Methods.

^bNumbers 1–4 denote the carbon numbers away from the ϵ -amino group, with the largest number being the most distant. The underlined carbons denote the ones with the given peak resonances.

^cThe control is the unmodified form of hippuryllysine (II).

GEL ELECTROPHORESIS OF MODIFIED MONOMERIC COLLAGEN

Mixing SSU with monomeric collagen in solution resulted in an increase in turbidity and viscosity. Analysis of the resulting mixtures were accomplished using SDS polyacrylamide gel electrophoresis, which separates the proteins according to their molecular weights by moving

the proteins through a porous gel matrix using an applied electric field. Ordinarily the larger the protein, the slower it moves through the gel. The migrations of α_1 and α_2 chains of collagen in SDS are, however, anomalous. Although their molecular weights are nearly identical, the α_2 chain migrates faster than α_1 (vide infra). Fig. 4 shows the results obtained from the analysis of modified monomeric collagen using gel electrophoresis.

The α and β bands of collagen are easily distinguished (see lane A). The α bands (α_1 and α_2) denote the two types of single-chain polypeptides that make up the collagen triple helix, whereas the β bands (β_{11} and β_{12}) are dimers consisting of two α_1 or one α_1 and one α_2 , respectively. We note that the modification of collagen with SSU (lane B) resulted in a higher mobility of the α_1 band as compared to the control (lane A), while the α_2 band remained relatively unaffected. In

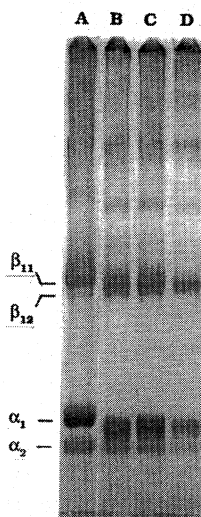


FIG. 4. — A 4% SDS polyacrylamide gel for collagen and SSU (Ia)-modified collagen is shown. The lanes are labelled as follows: A) control collagen, B) SSU (Ia)-modified collagen, C) SSU (Ia)-modified collagen extracted with ether, and D) SSU (Ia)-modified collagen extracted with chloroform/methanol (2:1). 25-30 μ g of collagen were loaded per well. The gel was run as described in Materials and Methods. The origin is at the top.

addition, compared to the control, the modified β_{11} band seems to have a slightly higher mobility (see lanes A and B).

Solvent extraction of the modified collagen with either ether or chloroform/methanol (2:1) does not appear to affect the anomalous mobilities of the modified α_1 or β_{11} bands (see Fig. 4, lanes C and D). In fact, aqueous reconstitution of the solvent extracted collagen generally returned them to their solution states prior to lyophilization and solvent extraction. In addition, thin layer chromatography of the solvent extract revealed an absence of any residual 10-undecenoic acid (data not shown). Furthermore, the increase in band mobility does not appear to be a result of protein degradation since no low molecular weight bands were observed below the α bands.

This type of anomalous band behavior has been previously observed by Furthmayr and Timpl²⁸ for the SDS polyacrylamide gel electrophoresis of methylated calf collagen. In fact, the α bands of collagen are themselves anomalous. Although the α_1 and α_2 polypeptides are of equal size, they differ in electrophoretic mobility, and both have mobilities lower than those observed for globular proteins of similar molecular weights^{28,29}.

Studies by Hayashi and Nagai²⁹ point to the low degree of hydrophobicity of the collagen polypeptides as an explanation for the anomalous mobilities³⁰. Using the free energies of hydrophobicity for amino acids, Hayashi and Nagai²⁹ showed that for their size, globular proteins have a higher degree of hydrophobicity than collagen polypeptides, i.e., globular proteins $>\alpha 2 > \alpha 1$. This is clearly shown in Table III. Although the calculated free energy values of the two sources differ slightly, they distinctly indicate that the hydrophobicities of $\alpha 1$ and $\alpha 2$, in comparison to globular proteins, are approximately one half of the expected value.

Since the binding of SDS to proteins is hydrophobic in nature, low hydrophobicity means low affinity for SDS. Therefore, the charge-per-mass ratio of the SDS-collagen complex is smaller than that expected for a globular protein of the same size. Consequently, the collagen polypeptides have anomalously low electrophoretic mobilities.

Any modification of the collagen polypeptides which leads to increased hydrophobicity would result in increased binding of SDS and higher electrophoretic mobilities of the polypeptides. Such is the case observed for methylated²⁸ and acylated collagen (see Fig. 4). On the other hand, succinylation of collagen decreases the hydrophobicity by increasing the surface polarity (and charge) of collagen, which in turn decreases its affinity for SDS. The resultant electrophoretic mobility of succinylated collagen polypeptides is lower than that observed for unmodified collagen²⁸.

TABLE III
FREE ENERGIES OF HYDROPHOBICITY FOR COLLAGEN
POLYPEPTIDES AND GLOBULAR PROTEINS

Protein ^a	# of Amino Acids	$\Delta G(\text{FP})^b$	$\Delta G(\text{EM})^b$
β -Galactosidase	1023	559.36	514.77
$\alpha 1(1)^c$	1000	104.09	183.52
$\alpha 2(I)^c$	1000	163.15	220.16
Phosphorylase B	841	399.41	380.31
BSA	581	225.03	222.67
HSA	585	222.42	226.62
Catalase	506	213.44	219.89
Pepsin	327	213.89	197.09

^a BSA and HSA denote bovine and human serum albumin, respectively. The amino acid sequences for the proteins were obtained from the Protein Sequence Database³¹ and the Handbook of Biochemistry and Molecular Biology³².

^b The free energy of hydrophobicity is given in kcal/mol. These values were calculated from the free energies of transfer for the individual amino acids. $\Delta G(\text{FP})$ and $\Delta G(\text{EM})$ denote the free energies determined from the values reported by Fauchere and Pliska^{35a} and Eisenberg and McLachlan^{35b}, respectively, for the individual amino acids.

^c The free energies for hydroxyproline and hydroxylysine were estimated from those for proline and lysine, respectively, using the free energy for OH (-0.66 kcal/mol for $\Delta G(\text{FP})$ and -0.76 kcal/mol for $\Delta G(\text{R})$).

TRANSMISSION ELECTRON MICROSCOPY OF COLLAGEN FIBRILS

The collagen fibril suspension had a slight turbid appearance, and, after reacting with SSU, there was a slight increase in turbidity. Transmission electron micrographs (Fig. 5) compare

untreated fibrils to the fibrils after interaction with the surface-active agent. These micrographs show qualitatively the presence of the surfactants, and whether any changes in surface texture and physical shape of the fibrils occurred due to the interactions with the surfactant.

Figure 5A shows the typical banding pattern of a positively stained collagen fibril from the untreated sample. On the other hand, Fig. 5B displays the collagen fibrils after reaction with the alkyl sulfosuccinimidyl ester. In this figure it is noted that the banding pattern is faint but apparently still intact and that there are no changes in the overall morphology of the fibrils. The surfactant employed here covers the fibrillar surface with an amorphous film. In negatively stained micrographs, the surfactant was generally seen to be present in a micellar form (micrographs not shown). In addition, preliminary scanning electron microscopy studies of treated wet-blue stock show that the surfactant is present on the surface of the exposed fibers in the blue stock, but does not appear to affect their structure (data not shown).

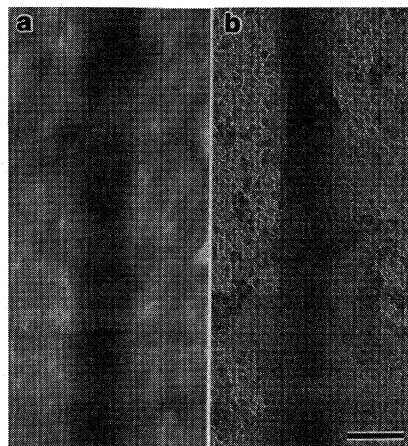


FIG. 5. — Transmission electron micrographs of positively stained collagen fibrils prepared as described in Materials and Methods: A) control fibrils and B) fibrils reacted with SSU (1a). Bar = 50nm.

Discussion

The reagents used in chemical modification and linking of proteins have been thoroughly reviewed^{20,29-32}. A major protein functionality that is commonly modified is the ϵ -amino group of such residues as lysine, histidine, and arginine. Next to the cysteine's sulfhydryl group, the ϵ -amino group is the second strongest nucleophile present. The reactive state for the ϵ -amino group is its unprotonated form, and thus most reactions are carried out at pH's approaching its pK_a , i.e., in the range $7 < \text{pH} < 9$ ³³.

Two common protein modifications are acylation and alkylation. Typical reagents used are diketene, imido esters and acetic and succinic anhydrides^{33,34}. Succinimidyl esters (e.g., 3,3'-dithiobis(succinimidyl propionate) were employed as more stable counterparts to the imido esters but they have a low degree of water miscibility^{31,35,36,37}. As a result, sulfonated derivatives were developed as more water soluble compounds^{20,36,38}.

The major advantages of the alkyl sulfo-N-succinimidyl esters are listed here. First, the reaction conditions are mild (i.e., pH 7.0 and room temperature), and the esters have been observed to have half lives over 2 hours^{36,38}. Second, they are reactive towards imidazole ring nitrogens, α and ϵ -amino groups, and to a lesser extent cysteine and tyrosine³⁸. In addition, although the rates for both the modifying action and the hydrolysis of these esters increase with pH and temperature, the modifying rate increases much faster than the hydrolysis rate³⁸.

Alkyl sulfo-N-succinimidyl esters (e.g., bis(sulfo-N-succinimidyl) suberate and sulfo-N-succinimidyl p-sulfobenzoate) have been employed as modifying or linking agents^{20,38}. Similar alkyl sulfo-N-succinimidyl esters have been used to modify and link proteins such as aldolase and the anion-exchange channel^{20,33}. In addition, these esters have been used to link platelet membrane proteins to collagen³⁹. As a result, these esters make suitable candidates as anchoring reagents for surfactants. Here, a 10-undecenoic acid derivative (Ia) was synthesized. The synthetic routes for its production and characterization are relatively straightforward. The product appears to be stable (mp = 227°C) and non-hygroscopic, but care must be taken to avoid yellowing due to oxidation of the double bonds. The solid has a distinct pungent odor, similar to that of lye soap. When dispersed in water or buffer solutions, the compound forms foamy, translucent mixtures, indicative of its surfactant properties. Similar characteristics were observed for N-alkyl- and N-acyloxymethyl-sulfosuccinimides²³.

The reactivity of alkyl sulfo-N-succinimidyl esters towards the ϵ -amino group of lysine has been shown to be second only to the imidazole ring nitrogen³⁸. The coupling reaction between SSU (Ia) and hippuryllysine (II) was studied using ¹³C NMR spectroscopy. The carbonyl carbon of the newly formed amide bond has a chemical shift downfield from the other amide carbonyls (see Table I). In addition, the presence of the new carbonyl deshields the carbons adjacent to the amide nitrogen, thereby shifting downfield their peak resonance values (see Table II).

From the electron micrographs and gel electrophoresis, it is apparent that SSU (Ia) coats collagen fibrils. Evidently, however, the interactions between SSU (Ia) and collagen are not solely due to adsorption but are also covalent in nature, since solvent extraction did not remove the acylating agent (see Fig. 5). Of the two α bands, the $\alpha 1$ is modified to a larger extent than $\alpha 2$. This may be a consequence of 44% more lysine residues in $\alpha 1$ than in $\alpha 2$ ³⁰. It is clear that this type of amino acid modification increases the hydrophobic character of collagen.

Surfactants have been postulated to interact with collagen through a two-step process, beginning with primary adsorption followed by secondary adsorption, resulting from direct hydrophobic interaction^{40,41}. Primary adsorption occurs initially from coulombic attraction between the surfactant and charged residues on the protein. This attraction permits the surfactant to approach the surface of the protein where hydrophobic forces come into play, which further bind the surfactant. Once primary adsorption has taken place, there is an increase in hydrophobic character in the area. Consequently, secondary adsorption occurs through direct hydrophobic interaction between the surfactant and protein. Apparently, secondary adsorption significantly enhances the adsorption of surfactants above what is expected according to electrostatic adsorption alone^{40,41}. Similar types of interactions are expected between the alkyl sulfo-N-succinimidyl esters (I) and collagen concomitant with any chemical reactions with exposed lysine residues. Our analyses show that the local binding is preferential and may be exclusive for the $\alpha 1$ collagen chain.

Conclusions

A reactive probe was used to locate possible binding sites for surfactants on collagen. The reagent coated collagen fibrils and reacted physically and covalently with the $\alpha 1$ chains of the collagen molecules. There was no evidence for binding to the $\alpha 2$ chains.

The surfactant properties of sulfo-N-succinimidyl esters (I) of long-chain carboxylic acids make them possible lubricants for leather that acylate collagen and thereby permanently increase its hydrophobic character. Their synthetic route and reaction conditions are straightforward. Furthermore, a crude sample of an oleic acid derivative has been synthesized using the procedure for the preparation of SSU (Ia), and the results from preliminary studies with this ester are similar to those observed for SSU (Ia). Studies on further effects of these esters on fibrillar collagen structure are in progress.

References

1. Benkiewicz, K.B. "*Physical Chemistry of Leather Making*", Robert E. Krieger Publishing Co. Inc., Malabar, Florida, 1983
2. Thorstensen, T.C., "*Practical Leather Technology*", 3rd ed., Robert E. Krieger Publishing Co. Inc., Malabar, Florida, 1984.
3. Heinrich, F., *Leather*, **193**, 66 (1991).
4. El A'mma, A., Hodder, J. and Lesko, P., *J. Am. Leather Chemists Assoc.*, **86** 1 (1991).
5. Tadros, Th. F. (ed.) "*Surfactants*", Academic Press Inc. (London) Ltd., London (1984).
6. Mital, K.L. and Lindeman, B. (Eds.), "*Surfactants in Solution*", Plenum Press, New York, (1984).
7. Tanford, C., "*The Hydrophobic Effect: Formation of Micelles and Biological Membranes*", 2nd Ed., John Wiley & Sons Inc., New York, (1980).
8. Fendler, J.H. & Tundo, P., *Acc. Chem. Res.*, **17**, 3 (1984).
9. Fendler, J.H., *Ind. Eng. Chem. Prod. Res. Dev.*, **24**, 107 (1985).
10. Meller, P., Peters, R. and Ringsdorff, H., *Colloid Polym. Sci.*, **267**, 97 (1989).
11. Hsu, C. and Berger, P.D., *J. Oil and Color Chemists Assoc.*, **73**, 360 (1991).
12. Andre, B., Boyer, B., Lamaty, G. and Roque, J-P., *Tetrahedron Lett.*, **32**, 1881 (1991).
13. Kuo, T. and O'Brien, D.F., *Langmuir*, **7**, 584, (1991).
14. Chu, D.Y. and Thomas, J.K., *Macromolecules*, **24**, 2212 (1991).
15. Caffrey, M., Hogan, J. and Rudolph, A.S., *Biochemistry*, **30**, 2134 (1991).
16. Laschewsky, A., Ringsdorff, H., Schmidt, G. and Schneider, J., *J. Am. Chem. Soc.*, **109**, 788 (1987).
17. Kippenberger, D., Rosenquist, K., Odberg, L., Tundo, P. and Fendler, J.H., *J. Am. Chem. Soc.*, **105**, 1129 (1983).
18. Reich, G. and Oertel, *Das Leder* **38**, 41 (1987).
19. Vogel, A.I., "*Vogel's Textbook of Practical Organic Chemistry*", 4th ed., Longman Scientific and Technical, Essex, London, 1987.
20. Staros, J.V., *Acc. Chem. Res.*, **21**, 435 (1988).
21. George, A. and Veis, A., *Biochemistry*, **30**, 2372 (1991).
22. Laemmli, U.K., *Nature*, **227**, 680 (1970).
23. Micich, T.J., Weil, J.K. and Linfield, W.M., *J. Am. Oil Chemists Soc.*, **52**, 451 (1975).
24. Andersen, G.W., Zimmerman, J.E. and Callahan, F.M., *J. Am. Chem. Soc.*, **86**, 1839 (1964).
25. Wehrli, F.W. and Wirthlin, T.W., "*Interpretation of Carbon-13 NMR Spectra*", Heyden & Son Ltd., London, 1980.
26. Lang, G.C., Lichter, R.L. and Nielson, G.L., "*Carbon-13 NMR Spectroscopy*", 2nd ed., John Wiley & Sons Inc., New York, 1980.
27. Kalinowski, H-O, Berger, S. and Braun, S., "*Carbon-13 NMR Spectroscopy*", John Wiley & Sons Ltd., New York, 1988.
28. Furthmayr, H. and Timpl, R., *Anal. Biochem.*, **41**, 510 (1971).
29. Hayashi, T. and Nagai, Y., *J. Biochem.*, **87**, 803 (1980).
30. Piez, K.A., In "*Extracellular Matrix Biochemistry*," (Piez, K.A. and Reddi, A.H., eds.), Elsevier, New York, 1984, pp. 61-67.
31. Protein Sequence Database, National Biomedical Research Foundation, Washington, DC.
32. *Handbook of Biochemistry and Molecular Biology: Proteins, Vols. II & III*, Fasman, G.D., ed., CRC Press, Cleveland, 1976.
33. Lundblad, R.L. and Noyes, C.M., "*Chemical Reagents For Protein Modification*", Vols. I & II, CRC Press Inc., Boca Raton, 1984.

34. Means, G.E. and Feeney, R.E., "*Chemical Modification of Proteins*", Holden-Day Inc., San Francisco, 1971.
35. (a) Fauchere, J.L. and Pliska, V., *Eur. J. Med. Chem. Chim. Ther.*, **18**, 369 (1983); (b) Eisenberg, D. and McLachlan, A.D., *Nature* 319, 199 (1986).
36. Staros, J.V., *Biochemistry*, **21**, 3950 (1982).
37. Lomant, A.J. and Fairbanks, G., *J. Mol. Biol.*, **104**, 243 (1976).
38. Anjaneyula, P.S.R. and Staros, J.V., *Int. J. Peptide Protein Res.*, **30**, 117 (1987).
39. Kotite, N.J., Staros, J.V. and Cunningham, L.W., *Biochemistry*, **23**, 3099 (1984).
40. Maldonado, F., Otero, A. and Almela, M., *Bol. Assoc. Quim. Esp. Ind. Cuero*, **40**, 199 (1989). *Ibid*, pp. 207-223.
41. Maldonado, F., Almela, M., Otero, A. and Costa-Lopez, J., *J. Protein Chem.*, **10**, 189 (1991).